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Two different types of humoral immune response to Actinobacillus actinomycetemcomitans in high-responder periodontitis patients

K. NAKASHIMA, C. USUI*, T. KOSEKI†, T. NISHIHARA† and I. ISHIKAWA*

Department of Periodontology, School of Dentistry, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-02, *Department of Periodontology, Faculty of Dentistry, Tokyo Medical and Dental University, Tokyo 113, and †Department of Oral Science, National Institute of Infectious Diseases, Tokyo 112, Japan

Actinobacillus actinomycetemcomitans is considered to be an aetiological agent in various forms of periodontitis, with serotype b-specific carbohydrate being the immunodominant antigen of A. actinomycetemcomitans Y4 in high-responder patients. Lipopolysaccharide (LPS) of the organism may also be an important antigen. The purpose of the present study was to clarify the importance of LPS as an antigen of A. actinomycetemcomitans. Twenty patients who had high antibody titres to strain Y4 were selected, and the reactivity of their sera with LPS was determined by ELISA and Western blotting. Two groups of patients were observed: group 1 had high IgG titres only to serotype b strain, whereas group 2 had high IgG titres to serotypes a, b and c strains. The results of adsorption tests showed that anti-A. actinomycetemcomitans Y4 antibody in group 1 patients mostly consisted of antibody reactive with the serotype b-specific carbohydrate, whereas the antibody in group 2 patients mostly consisted of antibody reactive with the LPS of all serotypes. These data show that anti-LPS antibody is present and predominant in anti-A. actinomycetemcomitans Y4 antibody from some high-responder patients, and indicate an important role for LPS as an antigen in the humoral immune response to the organism.

Introduction

Actinobacillus actinomycetemcomitans is a non-motile, gram-negative coccobacillus, which is considered to be an aetiological agent in localised juvenile periodontitis (LJP) [1] and severe adult periodontitis (AP) [2, 3]. A. actinomycetemcomitans has been also recognised as capable of causing other serious infections in man, including soft tissue abscess and endocarditis [4]. The organism was classified initially into three serotypes (a, b and c), and two additional serotypes (d and e) have been reported. Serotype b strains are isolated from LJP patients more frequently than are other serotype strains, suggesting a high periodontopathic potential for these strains that possess many virulence factors, e.g., serotype b-specific carbohydrate, lipopolysaccharide (LPS) and leucotoxin. The LPS and the serotype b-specific carbohydrate can stimulate macrophages to secrete various inflammatory mediators, including prostaglandins and interleukin-1 (IL-1) [5, 6]. These monocyte products are known to stimulate osteoclastic bone resorption, indicating that these bacterial components play an important role in periodontal bone resorption.

A previous study demonstrated that serum IgG titre to LPS from A. actinomycetemcomitans was significantly elevated in LJP patients [7]. However, it was not clear whether the elevated antibody titre was due to high levels of antibody to LPS itself or not, as the LPS prepared from A. actinomycetemcomitans Y4 by the phenol-water method also contains the serotype b-specific carbohydrate which is the immunodominant antigen in high-responder patients [8, 9]. This mixed preparation can be separated into a high-M, component (serotype b-specific carbohydrate) and a low-M, component (LPS) after gel filtration chromatography [8, 10, 11]. Page et al. have reported that the LPS (the low-M, component) was relatively non-immunogenic in high-responder patients [11]. Monoclonal antibodies (MAbs) specific for LPS have been developed, and the LPS was shown to be immunogenic in mice [8]. This raised the possibility that LPS could also be immunogenic in high-responder patients as well as the serotype b-specific carbohydrate. Therefore, the purpose of this study was to clarify if antibodies to...
Serum samples obtained. Each serum sample was measured for IgG titre; the study was explained to each patient and consent was obtained. Each serum sample was measured for IgG titre to A. actinomycetemcomitans Y4, and stored at −20°C until use. The mean IgG titre to A. actinomycetemcomitans Y4 was previously determined as 8.03 SD 2.36 for 28 individuals with healthy periodontal tissue [12]. In the present study, a high IgG titre to A. actinomycetemcomitans Y4 was defined as >1 SD above the mean IgG titre (i.e., >10.39). Twenty sera with high IgG titres were selected from >1000 stored sera, regardless of a clinical diagnosis. The patients were all Japanese, aged from 3 to 73 years and included two female patients (aged 3 and 7 years) suffering from Papillon-Lefèvre syndrome [13]. In some experiments, two MAbs (MAb S5 specific for the serotype b-specific carbohydrate, and MAb L2 specific for LPS from A. actinomycetemcomitans) were also employed [14].

Bacterial preparations

Three strains of A. actinomycetemcomitans (SUNYab 75, Y4 and SUNYab 67), which represent serotypes a, b and c, respectively, were obtained from the American Type Culture Collection. The bacteria were grown in Todd-Hewitt Broth (Difco) supplemented with yeast extract (Difco) 1% at 37°C for 3 days in a CO2 incubator (CO2 5% in air). Crude phenol-water extract was prepared from lyophilised whole cells of the three serotypes by the method of Westphal and Jann [15]. The crude extract was lyophilised, treated with nuclease (Nuclease P1; Wako Pure Chemicals, Osaka, Japan) and used as the phenol-water extract.

The phenol-water extract prepared from strain Y4 was further purified by gel filtration on Sephadex G-200 (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) in the presence of deoxycholate (Wako Pure Chemicals) [16]. A high-Mr component and a low-Mr component were eluted, as described previously [8]. Each component was lyophilised and then suspended in sterile pyrogen-free water (2 mg/ml). A 1-ml volume of the suspension was applied to a column (1.8 x 20 cm) of polymyxin B beads (Affi-Prep® Polymyxin matrix; BioRad Laboratories, Hercules, CA, USA), equilibrated with phosphate-buffered saline (PBS, pH 7.4) and washed with 10 ml of PBS (unbound fractions), followed by 10 ml of 1 N NaOH (bound fractions) [17, 18]. All fractions were measured for total sugar by the method of Dubois et al. [19].

Adsorption test

Type H beads for ELISA (Sumitomo Bakelite, Tokyo, Japan), 6.35 mm in diameter, were coated with the serotype-specific carbohydrate or LPS (1 mg/ml) by incubation at room temperature for 1 h. These beads were washed with PBS containing Tween 20 0.05% (PBST) and the unoccupied sites were blocked by incubation with PBST containing bovine serum albumin (BSA; Sigma) 1% for 2 h. The beads were then transferred to a microtube containing 0.5 ml of patient's serum. After incubation for 3 h, the serum was removed and stored at −20°C until use.

Enzyme-linked immunosorbent assay (ELISA)

Sonicate antigen was prepared from three serotypes of A. actinomycetemcomitans as described previously [12]. Briefly, bacterial cells disrupted by ultrasonication were centrifuged at 10,000 g for 20 min, and the supernate was dialysed against distilled water, lyophilised and used as the sonicate antigen. The microtitration plates (EIA/RIA Plate 3590; Costar, Cambridge, MA, USA) were coated with the sonicate antigen prepared from one of the three serotypes (10 µg/ml) suspended in 0.1 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. After blocking unoccupied sites with PBST containing BSA 1% for 1 h, serial two-fold dilutions of a patient's serum (1 in 32 to 1 in 32 768) were added to the plates. After incubation for 2 h, unbound antibodies were washed with PBST, and alkaline phosphatase-conjugated goat anti-human IgG (y-chain specific; Sigma) was added. The plates were incubated for 1 h, washed with PBST and p-nitrophenyl phosphate (Sigma) 1 mg/ml dissolved in 0.1 M carbonate-bicarbonate buffer was added. After incubation for 30 min, the absorbance was measured at 410 nm by a microplate reader MR-600 (Dyneach Laboratories, Chantilly, VA, USA). Incubation was at room temperature except for the antigen coating stage. The IgG titre was defined as log2 of the dilution that intersected at the 0.8 absorbance unit level, as described previously [12]. Samples were run in triplicate and measurement was performed twice.

Electrophoresis and Western blotting

SDS-PAGE was performed by the method of Laemmli [20]. Samples (5 mg/ml) were heated at 100°C for 5 min in SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8, containing SDS 2%, 2-mercaptoethanol 5%, glycerol 10% and bromophenol blue 0.025%; all reagents were purchased from Wako Pure Chemicals). The stacking gel contained acrylamide (Wako Pure Chemicals) 5%, and the separating gel contained acrylamide 12.5%. Electrophoresis was performed at a constant current of 20 mA at 10°C. The gel was stained with the ammoniacal silver reagent of Tsai and Frasch [21]. Samples separated by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes by the method of Towbin et al. [22] at a
constant voltage of 10 V overnight at 10°C. The membranes were incubated with a 1 in 100 dilution of a patient's serum for 1 h after blocking with PBST containing BSA 1%. The membranes were incubated for 1 h with a 1 in 1000 dilution of peroxidase-conjugated goat anti-human IgG (y-chain specific; Sigma) and washed with PBST. The membranes were soaked in a substrate solution containing 5 ml of 4-chloro-1-naphthol (3 mg/ml in methanol; Wako Pure Chemicals), 25 ml of 12 mM Tris-HCl (pH 8.0) and 10 μl of H2O2 30% v/v, then rinsed with distilled water and dried. Electrophoresis and Western blotting were performed twice for each sample.

Results

The phenol-water extract from *A. actinomycetemcomitans* Y4 was separated into high-Mr and low-Mr components by gel filtration. After affinity chromatography with polymyxin B beads, the high-Mr component showed a large and a small peak in the unbound and bound fractions, respectively, while the low-Mr component showed only a large peak in the bound fractions (Fig. 1). Unbound fractions of the high-Mr component were employed as the serotype b-specific carbohydrate as MAb S5 reacted with these fractions but not with the bound fractions. The low-Mr component was used as LPS from Y4 strain, as MAb L2 reacted with this component.

Twenty sera from high-responder patients were measured for IgG titres to antigens from serotypes a, b and c. Two different groups were observed (Table 1); group 1 patients (11 of 20) had significantly higher IgG titres to serotype b than to the other serotypes (p < 0.01, Wilcoxon signed-ranks test). Group 2 patients (9 of 20) had similar titres to all three serotypes. Group 1 patients consisted of nine patients with adult periodontitis (AP) and two patients with early-onset periodontitis (EOP), and group 2 patients consisted of four AP patients and five EOP patients.

![Fig. 1. Polymyxin B affinity chromatography of the high-Mr (-O-) and low-Mr (-○-) components, which were obtained by gel chromatography of the phenol-water extract prepared from *A. actinomycetemcomitans* Y4 on Sephadex G-200.](image_url)

| Table 1. Patient profile and IgG titres to three serotypes of *A. actinomycetemcomitans* |
|---------------------------------|-----------------|-----------------|-----------------|
| Patient no. | Sex | Age | Diagnosis | SUNYab 75 (a) | Y4 (b) | SUNYab 67 (c) |
| Group 1 | | | | Mean (SD) IgG titre to |
| 2 | F | 50 | AP | 9.94 (0.16) | 12.22 (0.22) | 8.43 (0.13) |
| 3 | F | 45 | AP | 7.96 (0.26) | 12.92 (0.32) | 9.16 (0.16) |
| 5 | F | 56 | AP | 9.69 (0.18) | 12.57 (0.20) | 8.77 (0.16) |
| 6 | M | 45 | AP | 6.94 (0.30) | 11.31 (0.31) | 7.37 (0.16) |
| 7 | F | 41 | EOP | 9.95 (0.13) | 13.54 (0.28) | 9.77 (0.17) |
| 9 | M | 44 | AP | 7.76 (0.28) | 12.95 (0.25) | 9.13 (0.18) |
| 14 | F | 47 | AP | 8.73 (0.12) | 13.50 (0.15) | 9.63 (0.13) |
| 15 | M | 35 | EOP | 8.51 (0.14) | 12.89 (0.18) | 9.01 (0.11) |
| 16 | F | 48 | AP | 8.73 (0.22) | 11.41 (0.13) | 7.62 (0.12) |
| 17 | F | 47 | AP | 9.16 (0.16) | 11.66 (0.20) | 7.82 (0.22) |
| 18 | F | 42 | AP | 9.41 (0.11) | 13.80 (0.33) | 9.02 (0.12) |

| Group 2 | | | | Mean (SD) IgG titre to |
| 1 | F | 3 | EOP | 11.18 (0.18) | 11.16 (0.10) | 11.14 (0.14) |
| 4 | F | 7 | EOP | 12.16 (0.26) | 12.03 (0.17) | 12.03 (0.23) |
| 8 | M | 73 | AP | 11.36 (0.25) | 12.60 (0.20) | 12.69 (0.29) |
| 10 | F | 38 | EOP | 10.87 (0.17) | 10.88 (0.28) | 10.94 (0.24) |
| 11 | M | 49 | AP | 11.53 (0.23) | 11.63 (0.23) | 11.65 (0.25) |
| 12 | F | 39 | AP | 11.49 (0.19) | 11.94 (0.16) | 12.10 (0.20) |
| 13 | F | 25 | EOP | 15.25 (0.35) | 10.90 (0.20) | 10.89 (0.29) |
| 19 | F | 43 | AP | 10.81 (0.21) | 13.83 (0.23) | 13.88 (0.38) |
| 20 | F | 26 | EOP | 10.54 (0.16) | 11.42 (0.22) | 11.28 (0.28) |

AP, Adult periodontitis; EOP, early-onset periodontitis. Titres are expressed as the mean (SD) of triplicate determinations. Comparisons 1 and 2 (p < 0.01, Wilcoxon signed-rank test); comparisons 3, 4, 5 and 6 (not significant, Wilcoxon signed-rank test).
The phenol-water extracts of the three serotypes were examined by SDS-PAGE and Western blotting. On the gel stained with the ammonical silver reagent, ladders were observed in all the phenol-water extracts, and a high-Mr smear was observed faintly only in the upper part of serotype b (Fig. 2A). Sera from group 1 patients reacted only with this smear zone of serotype b, but no interactions with the ladders of serotypes a, b and c were noted (Fig. 2B). A similar smear zone was also observed on a Western blot probed with MAb S5 (data not shown). Sera from group 2 patients reacted with the ladders of all the serotypes, but not with the high-Mr smear (Fig. 2C). Similar ladders were also observed on a Western blot probed with MAb L2 (data not shown).

Four representative sera, which had typical profiles on Western blot, were selected from each group for the adsorption test (Table 2). IgG titres to the sonicate antigen of strain Y4 were decreased markedly for group 1 after adsorption with the serotype b-specific carbohydrate (p < 0.01, paired t test), and only slightly decreased after adsorption with the low-Mr component (p < 0.05). When the differences in the titres obtained after adsorption with these two components were compared this was found to be significant (p < 0.01). However, the IgG titres were decreased slightly for group 2 sera after adsorption with the serotype b-specific carbohydrate (p < 0.05), and markedly decreased after adsorption with the low-Mr component (p < 0.01). When the differences in titre obtained after adsorption with these two components were compared this was found to be significant (p < 0.01).

Discussion

In subjects infected with A. actinomycetemcomitans, serum antibodies to the same serotype antigen of the infecting strain were detected, indicating a direct correlation between the colonising strain serotype detected and the systemic antibody response [23]. Ebersole et al. also reported the distribution of elevated IgG titres in A. actinomycetemcomitans-infected patients with periodontitis, demonstrating that 42.1% of the patients had high IgG titres only to serotype b, while 29.6% of the patients had high IgG titres to serotypes a, b and c [24]. They also reported that serotype b was the most frequent serotype detected and it appeared to be capable of initiating a substantial IgG serum antibody response, along with cross-reactive antibodies to other serotypes. In their study, 85% of the patients who had high IgG titres to serotype b may be classified into either group 1 (high titre to serotype b) or group 2 (high titres to all serotypes). The present study designated patients in a similar way and attempted to clarify the character of sera from group 1 and 2 patients by adsorption tests and Western blot analysis.

IgG titres to A. actinomycetemcomitans Y4 decreased markedly to a level similar to that in healthy individuals (i.e., c. 8.03) only after the sera were adsorbed with the serotype b-specific carbohydrate. When sera were adsorbed with the low-Mr component, titres ranged from 11.98 to 12.88 and remained at least 1 SD above the mean IgG titre for the healthy individuals. The slight but significant decrease in titre after adsorption with the low-Mr component may be due to non-specific adsorption or to the presence of a small amount of anti-low-Mr component antibody. Therefore, it seems likely that high IgG titres to serotype b sonicate for group 1 patients mainly result from antibody reactive with the serotype b-specific carbohydrate. The serotype b-specific carbohydrate is a polymer consisting of a disaccharide repeating unit, -(3)-α-D-frucopyranosyl-(1-2)-β-L-rhamnopyranosyl-(1-[25]. Wilson and Schifferle [10] and Page et al. [11] suggested that the serotype b-specific carbohydrate epitopes were located in the polysaccharide side chain (O-chain) of LPS. Recently, Perry et al. also demonstrated that the carbohydrate is O polysaccharide of LPS, consisting of a repeating trisaccharide unit composed of L-rhamnose, D-fructose, and G-galactosamine residue (1:1:1) [26]. Other workers have reported that the serotype b-specific carbohydrate is a capsular polysaccharide-like antigen, but different from the polysaccharide moiety of LPS [8]. In general, O-specific chains of LPS vary greatly in size and sugar composition, and various numbers of repeating oligosaccharide subunits account for the ladders observed on SDS-PAGE gels. In the present study, the low-Mr component appeared as the ladders on SDS-PAGE gels (Fig. 2A), indicating that the component contains repeating O-specific chains. The low-Mr component was also bound to polymyxin B (Fig. 1), indicating that the component contains lipid A. The low-Mr component is able to stimulate P388D1 cells to release interleukin (IL)-1, and this release was inhibited by polymyxin B [27]. These findings help to confirm that the low-Mr component is LPS.

In group 2 patients, high IgG titres to serotype b sonicate are due mainly to antibodies reactive with LPS, as IgG titres were markedly decreased only when the sera were adsorbed with the low-Mr component, but not with the serotype b-specific carbohydrate (Table 2). Koga et al. [8] reported that MAb L2 reacted with LPS (three strains for each serotype) from serotypes a, b and c in ELISA, indicating the presence of a cross-reactive antigen. The ladders observed on Western blots probed with sera from group 2 patients were similar to those on a Western blot probed with MAb L2. All sera from group 2 patients reacted with LPS from the serotype a, b and c strains (Fig. 2C). These findings suggest that group 2 patients have antibody reactive with an antigen contained in LPS, which is common to all the three serotypes. It remains to be determined if...
Fig. 2. SDS-PAGE and Western blot analysis of the phenol-water extracts. A, silver-stained gel: lane 1, SUNYab 75 (serotype a); 2, Y4 (serotype b); 3, SUNYab 67 (serotype c). B, Western blots probed with group 1 sera (nos. 3 and 9). C, Western blots probed with group 2 sera (nos. 1 and 4). Assignments of lanes in B and C are same as those in A.
the antibody is reactive with LPS from serotypes d and e.

Recently, Yamaguchi et al. reported that a MAb (MAb S5) specific for the serotype b-specific carbohydrate effectively opsonised *Actinobacillus actinomycetemcomitans* Y4 in killing assays and in in-vitro chemiluminescence assays [28]. They also described four mutant strains which lacked the serotype b-specific carbohydrate of the parent strain Y4. The mutants were more easily phagocytosed by human polymorphonuclear leucocytes (PMNLs), suggesting that the resistance of *Actinobacillus actinomycetemcomitans* to complement-mediated killing by human PMNLs might be attributed to the presence of the carbohydrate antigen. Therefore, the antibody to the serotype b-specific carbohydrate found in group 1 high-responder patients may opsonise *Actinobacillus actinomycetemcomitans* to prevent infection with the organism.

In conclusion, the present study shows that there are two major groups of patients who develop high antibody titres to *Actinobacillus actinomycetemcomitans* Y4. Group 1 patients have a large amount of serotype b-specific antibody which may be effective in preventing infection with serotype b strains of the organism. The large amount of serotype b-specific antibody probably indicates current or past infection with serotype b strain. Group 2 patients have a large amount of anti-LFS antibody. LPS from *Actinobacillus actinomycetemcomitans* has a broad spectrum of immunobiological and endotoxin activities, including mitogenic and polyclonal responses of B lymphocytes, macrophage activation and induction of IL-1 and prostaglandin E2 [8]. Also, the LPS is considered to be a potent mediator of alveolar bone resorption, which is one of the major features of periodontitis [5]. The high levels of anti-LPS antibody in group 2 patients may be effective in inhibiting immunological activity and preventing the progression of periodontitis. The present study should be regarded as a first step to clarify the exact roles of antibodies reactive with different antigenic components of *Actinobacillus actinomycetemcomitans* in the humoral immune response of patients with periodontitis and other infectious diseases.

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### References

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